analyse the expression of thousands of genes in parallel (Ye et al. 2001).

The last decade of research in environmental microbiology was an exciting period. It was dominated by the development and use of molecular biological methods to determine bacterial diversity and the structure of microbial communities. The coming decade, however, will even be more thrilling. Research will focus on ecological role of microbial diversity. The revolutionary developments in Life Sciences, such as whole genome sequencing and DNA microarrays will boost the field of environmental microbiology like molecular biological techniques did before. For the first time it will now be possible to determine the role of individual populations in complex mixtures of microorganisms by studying the expression of genes encoding enzymes that play a key role in the cycling of chemical elements, such as carbon, nitrogen and sulfur. However, it must be emphasized that only a *polyphasic approach,* in which concepts and methods from different disciplines, such as microbiology, ecology, molecular biology, and genomics, are combined, will reveal the role of microbial diversity in maintaining ecosystem Earth.

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OVERVIEW ON EXISTING MOLECULAR TECHNIQUES WITH POTENTIAL INTEREST IN CULTURAL HERITAGE

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Molecular Biology has provided with important techniques for studying cultural heritage and some of them might be of potential interest for the study and analysis of cultural assets. Molecular techniques complement more classical methods in the study of microorganisms and the fusion of these strategies is likely to result in a better understanding of the number, activity, and function of microorganisms in our cultural heritage.

Currently, there are large number of methods in use for the analysis of microorganisms on cultural assets, mostly involving cell counting and biodiversity analysis. Among these techniques the molecular methods attract special attention. Molecular Biology allows for culture-independent studies of the microorganisms in a sample. Microbial culturing is always problematic since most microorganisms in any sample either are in

dormant physiological stages or are unable to grow on the provided culture medium. Besides, molecular techniques take advantage of the specificity provided by the nucleic acid sequences and these methods are being developed during the last years, mainly in fields like the health sciences. Some of these methods can be easily adapted to the study of microorganisms in cultural heritage and their use is expected to experience a great increase in the next few years (Table 1).

Molecular techniques allow studying microorganisms from their DNA, RNA, and proteins. From the DNA, one can identify specific genes or microbial species. Since the quantity of RNA in a cell is proportional to its metabolic activity, the RNA provide with functional information based on the nucleic acid sequence. Proteins can also be used for functional studies and can be detected from their amino acid sequence, by using antibodies, and by direct activity measurements with enzyme assays.

DNA amplification is required to obtain high numbers of DNA fragments of interest from the tiny samples we have to analyze. Both DNA and RNA can be utilized since reverse transcriptase can be used to synthesize DNA from RNA. It is not widely known that there are different available techniques for DNA amplification: PCR (polymerase-chain reaction), LCR (ligase-chain reaction), and RCA (rolling-circle amplification). At present, PCR is by far the most used method for DNA amplification. There are continuous improvements. Taq DNA polymerase was the original thermostable polymerase, today there are several polymerases commercialized; for example, Pfu polymerase provides high 3´-proofreading activity so the number of errors during amplification is lower than with Taq polymerase. Today, it is common the use of polymerase blends consisting in combining polymerases to obtain the best characteristics from each of them. Also, several companies offer antibodies anti-taq, so that the taq polymerase is kept inactive until the antibody denatures during the first denaturing step of the PCR, in this way the synthesis of unspecific product previous to the thermal cycling of the PCR is avoided. Other improvements are the use of compounds to enhance the reaction or avoid inhibition; examples are the use of PVP, betaine, DMSO, PEG and many others. Other advances include multiple PCR and long PCR. Multiple PCR permits the amplification of different DNA fragments in the same reaction with different primer pairs. Long PCR allows the amplification of long DNA fragments; 50 Kb can easily be amplified using long PCR techniques.

Table 1. Perspective of use of molecular techniques in cultural heritage studies, most common current or potential applications, and increase of usage as an expected trend from a short- (S), medium- (M), or long- (L) term.

Technique	In use?	Most common Current or Potential Application	Usage trend*
DNA	Yes	Bacterial identification	S
RNA	Rare	Gene expression	S
Proteins	Rare	Activity through enzyme assays	M
PCR	Yes	DNA amplification	S
LCR	No	Detection of sequences and species	M
RCA	No	Detection of sequences and species	M
PCR enhancers	Yes	Avoid PCR inhibitors	S
Molecular beacons	No	Use as DNA probes	M
PNA	No	Improve properties of DNA probes	M
Multiplex PCR	No	Multiple reactions in a tube	M
Long PCR	No	Retrieving long DNA fragments	M
DGGE	Yes	Community profiles/Biodiversity	S
TGGE	Yes	Community profiles/Biodiversity	-
t-RFLP	Yes	Community profiles/Biodiversity	S
SSCP	No	Community profiles/Biodiversity	-
DNA library construction	Yes	Community profiles/Biodiversity	S
Cloning	Yes	Step previous to DNA sequencing	S
DNA Sequencing	Yes	Aimed to bacterial identification	S
FISH (microscopy)	Yes	Species-specific detection	S
FISH (flow cytometry)	No	Automated detection of species	M
FISH/MAR	No	Counting and activity of species	M
Quantitative PCR	Rare	Relative quantification of species	S
Quantitative RT-PCR	Rare	Quantification of gene expression	S
Web-based bioinformatics	Yes	Homology searches	S
Computer programming	Rare	Author-specific requirements	L
Molecular Databases	Yes	Homology searches	S
Genomics	No	Pathway analyses	L
Functional Genomics	No	RNA-based differential analyses	M
Proteomics	Rare	Differential protein analyses	M
DNA Microarrays	No	Quantifying multiple genes and species	M
Sample microarrays	No	High-throughput analyses	L
Genetic Engineering	Rare	Experimental gene expression studies	S
Reporter genes (e.g., GFP)	No	In situ gene expression analyses	S

* S, short-term; M, medium-term; L, long-term; -, increase of usage is not expected.

LCR uses a thermostable DNA ligase to ligate two oligonucleotides. If the sequence around the ligating site does not match, there is no ligation; if the sequence matches, the oligonucleotides will be ligated and this double oligo can be used as template leading to the exponential production of double oligos than can be easily detected. This reaction requires four

oligonucleotides; two of them need to be 5´-phosphorylated since it is a requirement for the ligation to occur. LRC can be used to discriminate sequences with differences of as low as a single nucleotide.

RCA does not require the use of a thermocycler since it is performed at a single temperature (30ºC). RCA uses bacteriophage Phi29 DNA polymerase which has an important property for this technique named strand displacement. This polymerase is able to displace the strand of DNA just synthesized so that it can continue synthesizing DNA from the template. The DNA template must be circular so if it is not, one should ligate it into a circular DNA template. This method with an appropriate primer design can be used to discriminate different sequences varying in a few nucleotides. Common PCR inhibitors appear to have little effect on RCA.

Other area of development is new **DNA labeling and detection methods**. Recently, highly fluorescent dyes are being commercialized. An example is SYBR Green I which shows high fluorescent only when binds double-stranded DNA. An elevated number of fluorescent dyes are being introduced for sequencing reactions (BigDye family), for DNA and RNA labeling (Cy5, Cy3), and many others (FAM, ROX, TAMRA, and many more) generally used for probe labeling.

Other strategies for probe design and labeling are the molecular beacons and PNA (peptide-nucleic acids). Molecular beacons are used for labeling DNA probes; a fluorescent dye is repressed by putting physically together a quencher with the fluorescent dye, when the probe binds to the target DNA fragment the quencher and dye separate and high fluorescence emission can be detected. The PNA are synthetic analogues to DNA with the backbone of sugar-phosphate replaced by a chain of N-(2-aminoethyl)-glycine linked with amide bonds. PNA are of great interest for DNA or RNA probe construction since they are more specific than complementary strands of DNA and the temperature of hybridization can be greatly increased.

Biodiversity or microbial community profiles are being used in cultural heritage studies on microorganisms to analyze the presence of diverse microbial species in a sample. Different techniques are available for this purpose, such as DGGE (Denaturing Gradient Gel Electrophoresis), TGGE (Temperature Gradient Gel Electrophoresis), t-RFLP (Terminal Restriction Fragment Length Polymorphism), SSCP (Single Strand Conformational Polymorphisms), or cloning and sequencing. DGGE is the most used profiling technique in cultural heritage studies. Basically, these profiling techniques require a DNA extraction followed by DNA amplification and the amplified products are then processed for the specific technique to be used and run following a specific gel electrophoresis protocol. Results are analyzed by visualizing the resulting bands or peaks corresponding to the amplified products. Each technique has its pros and cons and investigators have their own preferences. These techniques can be complemented with cloning and sequencing in order to identify the bands to specific microbial species by comparison with information obtained from DNA databases.

FISH (Fluorescent In Situ Hybridization) is a technique attracting great interest in the last years, and permits the visualization of specific microbial cells in complex communities. By FISH one can detect specific genes using an oligonucleotide probe generally labeled with fluorescent dyes. The labeled cells can be visualized under a microscope or using a flow cytometer. In the last years, FISH has been combined with the uptake of radiolabelled substrates and microautoradiography in a pretty complete method, FISH/MAR, that allows counting and activity measurements of specific microorganisms.

Quantification of DNA and RNA is now possible utilizing quantitative, real-time PCR techniques. The amplification of DNA (or RNA) is monitored during the thermal cycling and different DNA concentrations will reach a DNA concentration threshold at a distinct number of cycles, which is used for estimating the initial DNA template concentration in each sample relative to another sample. If an absolute estimation

of DNA concentration is required, one can prepare a calibration curve with reactions of known DNA concentrations. This technique shows high accuracy and expands for up to 5 orders of magnitude. Other alternatives for the quantification of DNA are also available such as Most-Probable Number-PCR (MPN-PCR), competitive PCR, or the use of FISH and flow cytometry.

Bioinformatics are of great importance when applying advanced molecular biology methods. A number of pieces of software are everyday used for alignments, phylogenies, or database search for mentioning a few cases. The importance of DNA databases is critical since we constantly refer to them for the identification of the microorganisms whose sequences have been detected in our studies. Generally, the most commonly used gene for identifying bacteria is the 16S ribosomal RNA. There are databases of DNA, ribosomal RNA gene sequences, protein sequence and structure, genomes and expression profiles.

Advances in **genomics, functional genomics and proteomics** are introducing a large number of possibilities and strategies for the analysis of microorganisms from cultural assets. Genomics deals with the structural analysis of DNA sequences, which can be used for multiple comparisons and in the search of information corresponding to certain metabolic pathways of interest, for instance, for the conservation of our heritage. Functional genomics deals with the functional analysis, generally based on RNA, of microorganisms by studying cell-wide, gene expression profiles. Functional genomics can be of great interest for the study of gene expression of microorganisms causing damage to specific cultural assets and the variability corresponding to different environmental conditions. Generally, functional studies use DNA microarrays or DNA chips to analyze thousands of genes on a microslide. The DNA chips can contain many species-specific probes for the identification of high number of bacteria or a large number of protein-encoding gene probes for obtaining gene-expression profiles of genes of interest for specific pathways of importance in cultural heritage. Proteomics open the door to similar studies based on proteins. In proteomics, researchers generally perform 2D-gels to visualize the proteins expressed in a sample or by a microorganism. Differential analysis allows detecting the proteins expressed at different levels under two distinct conditions.

A step forward would be the construction of microarrays of samples. These sample microarrays would contain large number of samples (by the thousands) that could be analyzed, for instance, through FISH techniques for the detection of specific microorganisms or the expression of specific genes.

Genetic engineering also provide with novel opportunities for cultural heritage. Novel cloning vectors and techniques, for instance, allow cloning specific proteins fused to reported genes such as the GFP (green Fluorescent Protein). These reported genes permit to easily know where that gene is expressed and quantify its expression since fluorescence can be easily measured.

Some of the above-mentioned techniques can be successfully applied to cultural heritage studies. Most of these methods are expected to be increasingly used in the next few years (Table 1). Hopefully, throughout the next years, we will expect a continuous and increasing funding from European programs into Molecular Biology projects on Cultural Heritage.

CONFOCAL LASER SCANNING MICROSCOPY AND IMAGE PROCESSING FOR ANALYSIS OF AEROPHYTIC BIOFILMS IN CULTURAL HERITAGE

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Aerophytic biofilms are formed by microorganisms and by the extracellular polymeric substances they produce and, in